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Singles Engage the RNA Interference Pathway

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Single-stranded RNAs interact with components of the RNA interference pathway to reduce the expression of target mRNAs. Now, Lima et al. and Yu et al. show that, with extensive chemical modifications, small single-stranded RNAs can robustly induce gene silencing with efficacy similar to their double-stranded counterparts.

Nucleic acids and chemical derivatives synthesized as antisense oligonucleotides (ASOs) or small interfering RNAs (siRNAs) are used widely to reduce the expression of target genes for functional assays and therapeutic purposes. ASOs are single-stranded oligonucleotides designed to base-pair with target RNAs and reduce their expression by triggering RNaseH-dependent or -independent mechanisms (Bennett and Swayze, 2010) (Figure 1A). siRNAs are double-stranded RNAs (dsRNAs) that engage the endogenous RNA-induced silencing complex (RISC) and repress expression by base-pairing with and cleaving target mRNAs via a mechanism that is dependent on the cofactor Ago2 (Figure 1B) (Davidson and McCray, 2011). Single-stranded siRNAs (ss-siRNAs) also induce gene silencing, but their potency is significantly lower than that of their dsRNA counterparts (siRNAs, short-hairpin RNAs [shRNAs], or artificial microRNAs [miRNAs]) (Schwarz et al., 2002). In this issue of *Cell*, two studies (Lima et al., 2012; Yu et al., 2012) show that chemical modifications to the ss-siRNA dramatically improve both potency and activity. The ss-siRNA activity requires RNAi pathway engagement and demonstrates target gene silencing in liver (Lima et al., 2012) and brain (Yu et al., 2012).

Endogenously expressed microRNAs (miRNAs) typically inhibit the expression of target mRNAs that contain sequences complementary to bases 2–8 of the antisense (or guide) strand of the miRNA known as the seed region (Lewis et al., 2005). Though siRNAs, shRNAs, and artificial miRNAs are intended to induce target transcript cleavage via perfect base-pairing, they can also mediate silencing of off-target mRNAs by seed-based recognition as with the canonical miRNA-like mechanism (Birmingham et al., 2006). And the sense strand, if loaded into the RNAi machinery, can behave similarly. This potential to induce substantial off-target silencing and toxicity is obviated by the ss-siRNA design; the sense strand does not exist. The antisense strand can still pose this risk, however, a concern that was not addressed in the current studies. Given the high doses delivered for reduction of target mRNAs in vivo, off-target gene silencing via miRNA-like mechanisms will require careful evaluation. For example, the CAG-repeat-targeting constructs used by Corey and colleagues (Yu et al., 2012), which show selectivity for mutant huntingtin over wild-type huntingtin, use ss-siRNAs with putative seed sequences that could silence numerous other transcripts that do not necessarily contain the full complement of the ss-siRNA.

A major advance toward the future utility of ss-siRNAs comes from the exhaustive investigation of numerous modifications to the ss-siRNA design and their impact on stability, pharmacokinetics, and potency (Lima et al., 2012). Of note, the authors confirm the importance of the 5′-phosphate for activity (Schwarz et al., 2002) and identify a stable 5′-phosphate modification using a 5′-(*E*)-vinylphosphate. The ss-siRNAs become associated with Ago2, and both studies show that the ss-siRNAs require Ago2 for activity. In the work by Crooke and colleagues (Lima et al., 2012), the ss-siRNAs are fully complementary to the target sequences, and Ago2 is required for cleavage. Intriguingly, Yu et al. (2012) design an ss-siRNA with a single mismatch between the target and the ss-siRNA, causing a bulge. Again, Ago2 is required for activity, but cleavage of the target transcript does not occur; a bulge in the sequence probably inhibits this activity. Interestingly, this form of translational repression does not ultimately cause target destabilization and a concomitant decrease in transcript levels as found for most mRNAs acted upon by endogenous miRNAs (Guo et al., 2010). This may reflect differences between miRNA-mediated silencing by either exogenously expressed miRNA sequences or endogenously expressed miRNAs, which most often occur via interactions with 3′-untranslated regions (Birmingham et al., 2006). The sequences employed by Yu and colleagues (Yu et al., 2012) target the first exon.

After testing of the ss-siRNAs in vitro, their therapeutic utility is examined in vivo against several targets in the liver (Lima et al., 2012) or in the brain (Yu et al., 2012). Potent gene silencing of Factor VII, PTEN, and ApoCIII is observed with ss-siRNAs in saline-based buffers in contrast to the usual requirement for lipid-based formulations for ds-siRNAs, which can often cause toxicity. Silencing activity is improved further by the addition of a lipophilic moiety on an internal base,

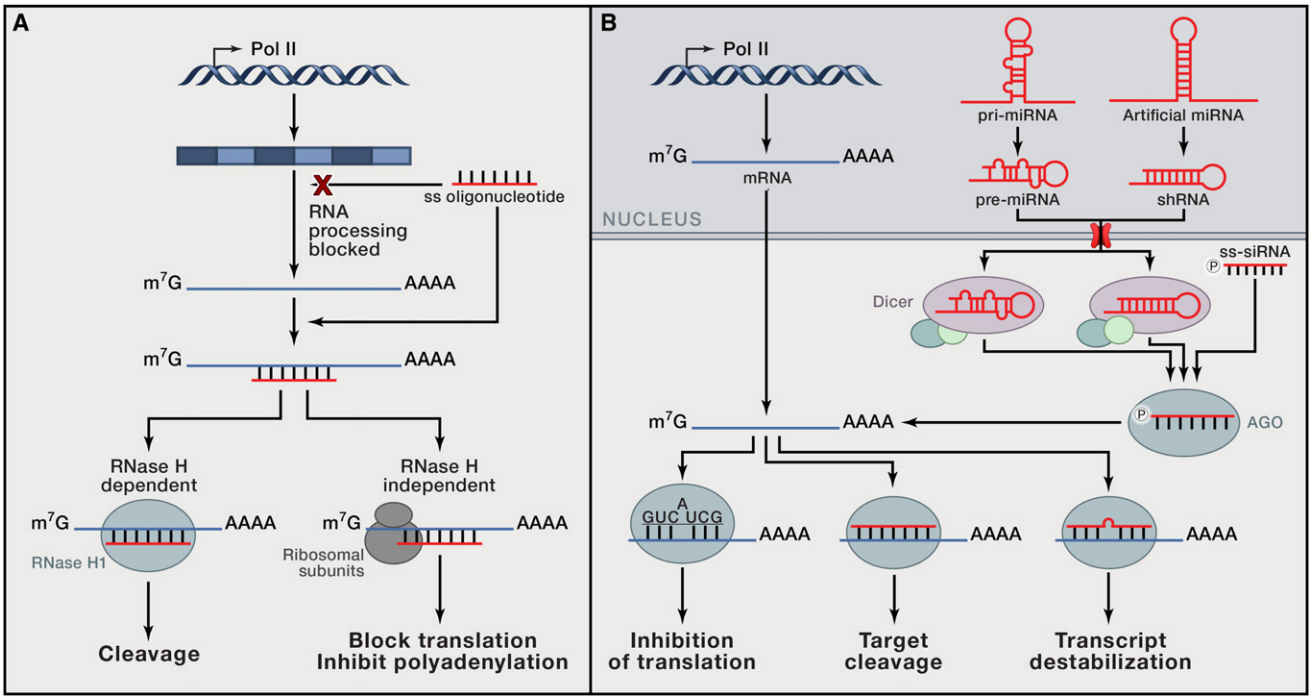

Figure 1. Gene Silencing Approaches in Mammals

(A) Antisense oligonucleotides (ASO) are designed to bind and reduce the expression of a target RNA by: (1) activating RNaseH protein and inducing RNA degradation or (2) acting as steric blockers and interfering with the maturation or translation of a target RNA.

(B) Small interfering RNAs (siRNAs) are designed to co-opt the RNA-induced silencing complex (RISC) of the endogenous RNA interference (RNAi) pathway to reduce the expression of a target RNA. siRNAs can be designed as single-stranded (ss-siRNAs) or double-stranded RNAs (siRNAs). Alternatively, double-stranded RNAs can be incorporated into expression vectors by embedding siRNA sequences into stem-loop structures designed to mimic endogenous primary miRNA transcripts (artificial miRNAs) or pre-miRNAs (short-hairpin RNAs [shRNAs]). Artificial miRNAs and shRNAs are incorporated into the RNAi pathway before or after the microprocessor complex stage (Drosha-DRG8 processing), respectively, and are then exported to the cytoplasm. For double-stranded siRNAs, one of the two strands is degraded. ss-siRNAs are directly incorporated into the RISC complex.

which improves serum stability and uptake (Lima et al., 2012). The target tested in the mouse central nervous system (CNS) is mutant huntingtin, the underlying cause of the fatal, autosomal-dominant neurodegenerative disorder Huntington's disease (HD). The mutation, a polyglutamine-coding repeat (>36 repeats) expansion in exon 1 of the huntingtin mRNA, provides one avenue for specific silencing of the disease allele while leaving the wild-type allele, which has fewer CAG repeats, intact. The therapeutic benefits of silencing mutant huntingtin expression with ASOs and inhibitory RNAs have been reported (Boudreau et al., 2009; Kordasiewicz et al., 2012) and are in preclinical testing. Yu et al. (2012) revisit CAG repeat targeting using ss-siRNAs harboring the chemical modifications found by Crooke and colleagues to improve in vivo stability and activity (Lima et al., 2012) of sequences previously tested as dsRNAs. Interestingly, the ss-siRNAs are more potent than their ds-siRNA counterparts. Similar to ASOs targeting huntingtin delivered into the mouse brain ventricles, the ss-siRNAs distribute widely throughout the brain and reduce mutant huntingtin protein levels (Kordasiewicz et al., 2012), but in this study, there is limited impact on the levels of wild-type protein. One caveat with the in vivo selectivity assay revolves around the fact that, with respect to human sequence that contains a stretch of 10 to 29 CAG repeats, the 7-glutamine repeat in exon 1 of the normal mouse allele is not encoded by a pure CAG repeat. Thus, the additional mismatch between the wild-type allele and the ss-siRNA could contribute to the enhanced selectivity of silencing in vivo.

Repetitive dosing of the ss-siRNAs to liver does not increase liver enzymes associated with hepatocellular toxicity. Similar assessments in the brain were not done. Given the doses applied to the CNS (10 mg/kg per day for 28 days) and the fact that ss-siRNAs engage the RNAi machinery, careful toxicity studies will be required in future work. Potential saturation of the RISC by ss-siRNAs, which in the setting of siRNA or expressed shRNAs and miRNAs can reduce the proper processing and activity of endogenous miRNAs, will also be important to investigate. The association of ss-siRNAs with Ago2 (and presumably the other Agos) may impact the normal function of these proteins in cells. As noted by Corey and colleagues, it will be difficult to infer the toxicity and off-target impact from their ds-siRNA counterparts, and thus directed studies on the ss-siRNAs themselves will be important.

In summary, these studies introduce ss-siRNAs as an exciting new tool in our arsenal to silence disease-causing genes for therapeutic applications and basic research. It is also possible, given their

stability and ability to enter cells without lipid formulation, that they may find utility as a sponge for disease-related miRNAs. Given the noted tolerance of the liver to high doses of the ss-siRNAs, they could conceivably be used to inhibit miRNAs involved in disease pathogenesis. Indeed, tiny locked nucleic acids (LNAs) with complementarity to miRNAs show promise in reducing miRNA activity (Obad et al., 2011).

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Holding on through DNA Replication: Histone Modification or Modifier?

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Histone methylation is widely believed to contribute to epigenetic inheritance by persevering through DNA replication and subsequently templating methylation of daughter chromosome regions. However, a report in this issue (Petruk et al.) suggests that chromatin association of the methytransferase complexes themselves persists through replication and re-establishes histone methylation.

Epigenetics is the study of heritable changes in gene expression caused by mechanisms other than changes in the underlying DNA sequence. Epigenetic inheritance of specific patterns of gene expression is essential for the maintenance of cell lineages. Large multiprotein complexes and posttranslational modifications of histone proteins that package DNA have been linked to both the active expression and repression of genes that define particular cell types. However, the mechanism by which this protein architecture is manipulated to allow the replication machinery to pass by but remember its original configuration for reassembly has been a topic of much study and debate. Covalent histone modifications have been implicated in epigenetic inheritance in numerous studies (Suganuma and Workman, 2011; Zhu and Reinberg, 2011), and it has been proposed that these modifications are maintained at specific genomic loci through mitotic cell divisions. A model has emerged in which modified histones on parental DNA are randomly partitioned to daughter strands during DNA replication and subsequently promote the modification of newly added histones. This mechanism would ensure that the architecture of specific loci is passed on to daughter cells (Corpet and Almouzni, 2009; Zhu and Reinberg, 2011). A provocative report now challenges, or at least provides a dramatic alternative to, this model. Petruk et al. (2012) present evidence from *Drosophila* embryos that histone modifications are actually lost during DNA synthesis and, instead, that the association of histone-modifying enzyme complexes with specific loci persists during replication and re-establishes the histone modifications after S phase.